Ethanol Tolerance of L1400 Yeast

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Researchers

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Objective

To determine the maximum tolerance of L1400 yeast to the inhibitory effects of ethanol and to investigate soy flour as an additive with the potential of improving yeast tolerance to ethanol.

Background

This continuous fermentation was conducted as part of ongoing bench-scale support to the PDU, in an effort to further optimize processes within the Biomass to Ethanol project. The determination of yeast cell tolerance to various fermentative inhibitors is essential data that must continue to be generated, as pilot plant scale-up and shakedown continue.

In order for the current distillation system to be economical, the processed fermentation broth needs to contain 7-8% ethanol. Because this concentration might not be attainable depending on the organism and feedstock being used, a recycle system might have to be employed to boost the ethanol concentration in the fermentations. This experiment was designed to look at the tolerance of exogenously added ethanol to a fermentation to simulate a recycle scenario in the PDU. The design includes the utilization of soy flour as a protective agent that has been cited in literature to increase ethanol tolerance.

Materials and Methods

A New Brunswick BIOFLO III fermentor was employed for the continuous fermentation. Overlay house air filtered to 0.2µm was delivered @ 1 vvm. To insure against evaporation, the fermentor's condenser was packed with 1mm glass beads and equipped with 4.0°C H_2O circulation. The working volume for the vessel was 750 ml. Agitation was set @ 150 rpm, and the reactor temperature was maintained @ 30.0°C. The pH was controlled at 5.0 by the addition of 3M NaOH or 3M H_3PO_4.

A frozen stock vial of the yeast *Saccharomyces diastaticus* (L14000, supplied by Amoco) was grown in YPD. 10% v/v was then transferred to 1% w/v corn steep liquor (CSL) and 2% w/v glucose for inoculum growth. The fermentation was started in batch mode with 1% w/v yeast extract, 2% w/v CSL, and 5% w/v glucose for medium. A 10% v/v inoculum was transferred to the vessel, and when the culture exhausted the initial glucose supply, the fermentation was switched to continuous mode.

The 10L feed carboy was composed of 1% w/v yeast extract, 2% w/v CSL and 5% w/v glucose. The CSL was prepared by initially diluting to 10% w/v, centrifuging, and

filtering with Gelman capsule filters in series(1.2µm 0.45µm 0.20µm). The pH of the feed was adjusted to 5.0 and autoclaved for 90-120 minutes. A 10L waste carboy was used to collect the outcoming fermentation broth. Both carboys were equipped with size 16 Masterflex silicone tubing, a Gelman Acrodisc air vent filter, and a syringe port for sampling of contents. Each carboy was placed on a balance, and weights were routinely recorded. Feed was pumped into the vessel and waste removed using high flow Watson-Marlow peristaltic pumps. The flow rates and weights were monitored to maintain specific dilution rates and residence times.

Once initial steady states were established, the external addition of 95% w/w ethanol began. The ETOH addition system was cycled through the existing 4.0°C H_2O bath, and a glycerol bath was **also** employed to further reduce evaporative **loss.** A Watson-Marlow low flow pump delivered the ETOH exogenously, while a balance kept track of weight changes during the experiment.

In order to determine the potential of improving the yeast cell tolerance in the presence of high ethanol concentrations during fermentation, soy flour was later added **as** a protective agent against product inhibition. A two fold concentration of feed was prepared and autoclaved in a 10L carboy with a stir bar. The soy feed was made as a 6% w/w solution and mixed with the feed in a laminar flow hood after being autoclaved separately to yield a final concentration of 3% w/v soy flour. The feed was mixed continuously with a stir plate situated on a balance.

The sampling protocol consisted of three samples per day. Weights from balances were initially taken, and calculations of % ETOH, residence time, and dilution rate were made. Effort was taken to use sterile sampling vials. For every sample, *two* aliquots were drawn. One was sterile and utilized in viability assays and microscopic analysis. Another aliquot was used to validate reactor pH, glucose and ETOH concentration by YSI, dry cell weight, and optical density. HPLC vials were also prepared and run **on** an organic acids column. A five ml sample was centrifuged, washed twice with 10 ml deionized water , and dried at 60°C for **24** hours to determine dry cell weights.

Results and Discussion

Six steady states were achieved over the 1000 hour continuous fermentation. Table 1 summarizes the conditions of each steady state.

Table 1

Steady State	Residence Time (h)	Ethanol In (% w/w)	Ethanol In* (g/L)	Soy flour in feed (% w/v)
1	11.56	0.00	0.00	
2	11.14	3.39	*33.63*	
3	11.12	6.13	60.52	
	10.89	8.96	88.10	
5	10.35	8.52	83.84	*3*
4	23.66	9.89	97.12	
6	21.49	17.15	166.75	3

* **Based** on **the density** of ethanol

In the first steady state ethanol was not added exogenously to the culture and 19.3 g/L ethanol was produced representing a theoretical yield of 75.7% (see fig. 1). The cell mass yield obtained for this steady state was 0.14 g/g **(see** fig. **2).** After the first steady state was completed, 3.4% w/w ethanol was added to the fermentation. This resulted in a decreased theoretical yield of 50.72% and a lowered cell mass yield of 0.12 g/g. The ethanol concentration added to the reactor was increased by another 3% w/w to 6.13% w/w for the third steady state. This again resulted in a lowering of the theoretical ethanol yield to 22.39% and a decreased cell mass yield of 0.08 g/g. In all three steady states all the glucose supplied (~50 g/L) was consumed.

A fourth steady state was attempted at the dilution rate of 11 hours and 9% w/w ethanol. Under this condition the residual glucose quickly increased , the OD plummeted, and we quickly approached washout conditions. At this point, the residence time was adjusted to 24 hours to determine if this residence time would allow recovery of the fermentation. At a residence time of **24** hours and almost 10% w/w ethanol we were able to achieve a steady state (#4) with a measured ethanol concentration of 70.15 g/L and no residual glucose. The cell mass yield was increased slightly to 0.10 g/g.

Because one of our objectives was to investigate the effect of soy flour on ethanol tolerance, we added 3% w/v soy flour to the feed for the next two steady states. Since we experienced washout conditions at a residence time of **12** hours and an ethanol concentration of 9% w/w we went back to those conditions to determine if tolerance would increase with the addition of soy flour. With the addition of soy flour we were able to achieve a steady state with 70.4 g/L ethanol in the fermentor. In the sixth, and final steady state, we added 17% w/w ethanol and measured 107.8 g/L in the fermentor. Due to the heterogeneous delivery of the soy flour feed, dry cell weight values were not a good indication of cell mass. However, hemacytorneter counts showed that yeast were still present in the fermentation even at concentrations of 107.8 g/L. Table 2 summarizes the results obtained for each steady state.

Table 2

Steady State	Residence Time (h)	Residual Glucose (g/L)	Ethanol measured (g/L)	Dry Cell Weight (g/L)	Cell Mass Yield (g/g)	Theoretical ETOH Yield (%)	ETOH Yield (g/g)
1	11.56	0	19.34	6.99	0.14	0.76	0.39
2	11.14	0	46.59	5.84	0.12	0.51	0.26
3	11.12	0.122	66.23	4.01	0.08	0.22	0.11
	10.89			wash out			
4	23.66	0.595	70.17	4.95	0.10	-106.75	-0.55
5	10.35	38.94	70.39			-237.99	-1.22
6	21.49	43.7	107.8			-1831.29	-9.36

Unfortunately dry cell weights did not give us an accurate reflection of the cell mass when soy flour was added to the feed due to the inability to deliver a homogeneouos feed mixture continuously even though we had it stirring on a stir plate. For future continuous work with soy flour, a better method should be utilized to enable the deliver of a homogeneous feed. The run was terminated at 1000 hours due to bacterial contamination in the **feed.**

Glycerol, lactic acid, succinic acid, and acetic acid, typical L1400 by-products, were also measured for by HPLC. The major by-product produced was glycerol (.5 to 1 g/L) with minor amounts of lactic acid and succinic acid produced (see fig 3). The feed consisted of ~2 g/L lactic acid that has not been taken into account in this chart. The feed did not consist of any other by-products. It is interesting to note that there is a possible cycling of lactic acid and glycerol production throughout the run. An increase in acetic acid production is seen at 700 hours when contamination was observed.

Dry cell weights and optical density (600 nm) were also determined on a regular basis. From this data we were able to get a good correlation of the g/L dry cell weight per OD measured, 0.6 g/L per OD unit, (see fig. 4). This value will be useful in the future to determine approximate dry cell weight values based on OD measurements,

Conclusions

As described in the materials and methods section, the nutrients for this fermentation were very rich consisting of 2% w/v CSL and 1% w/v yeast extract. A previous experiment was attempted with just 2% w/v CSL, the yeast morphology changed significantly forming extended pseudo-mycelia. The addition of 1% w/v yeast extract in the second attempt decreased the extent of hyphal formation. Even though CSL is a fairly rich source of nutrients, it might be deficient on certain elements affecting growth under the operating conditions. With this high level of nutrients, we observed a maximum ethanol tolerance of *6.6%* w/w with a residence time of 12 hours. If the residence time is increased to 24 hours, the tolerance increased to 10% w/w ethanol. With the addition of 3% w/v soy flour, a 2% w/w increase in tolerance to *8.5%* w/w was observed with a residence time of 12 hours.

Upon examining the data, it is apparent that a large amount of carbon is unaccountable. This loss could be due to evaporative losses of ethanol in the system even though precautions were taken to minimize these losses. The other possible area where it could have been unaccounted for is through the production of CO, by means of ethanol utilization. It is known that under certain conditions S. *cerevisiae* can simultaneously utilize glucose and ethanol in continuous fermentations (Jong-Gubbels, 1995). Unfortunately the mass spec was not operational during this experiment. In further studies CO, and evaporative losses of ethanol should be measured in some manner.

Figure 1
Ethanol and Glucose Concentrations

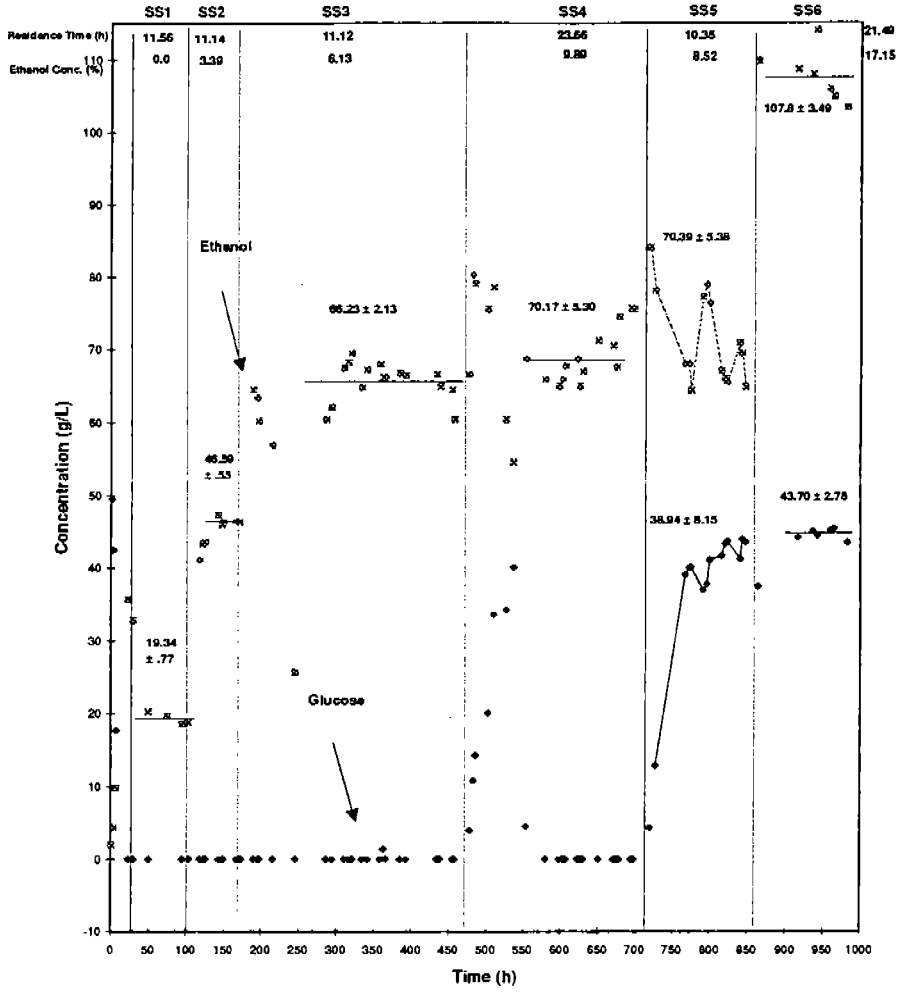


Figure 2
Dry Cell Weights

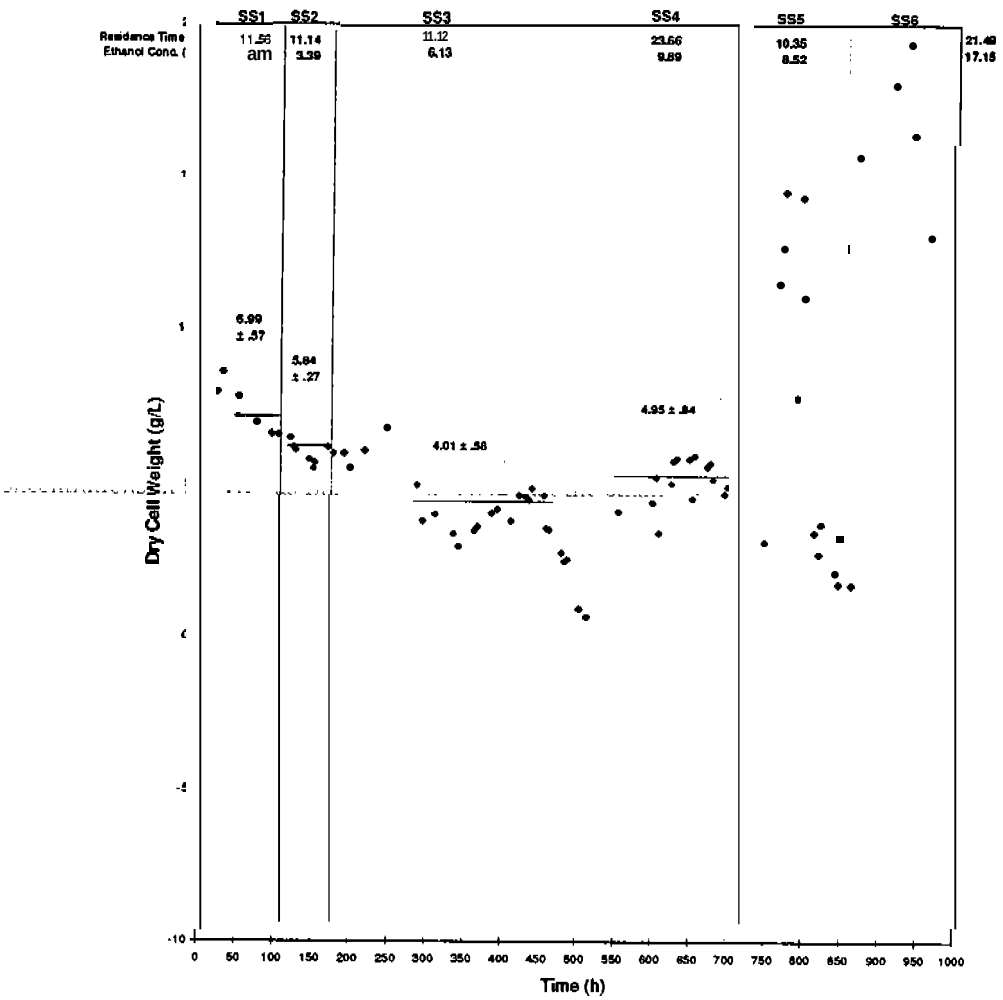


Figure 3
By-product Concentrations

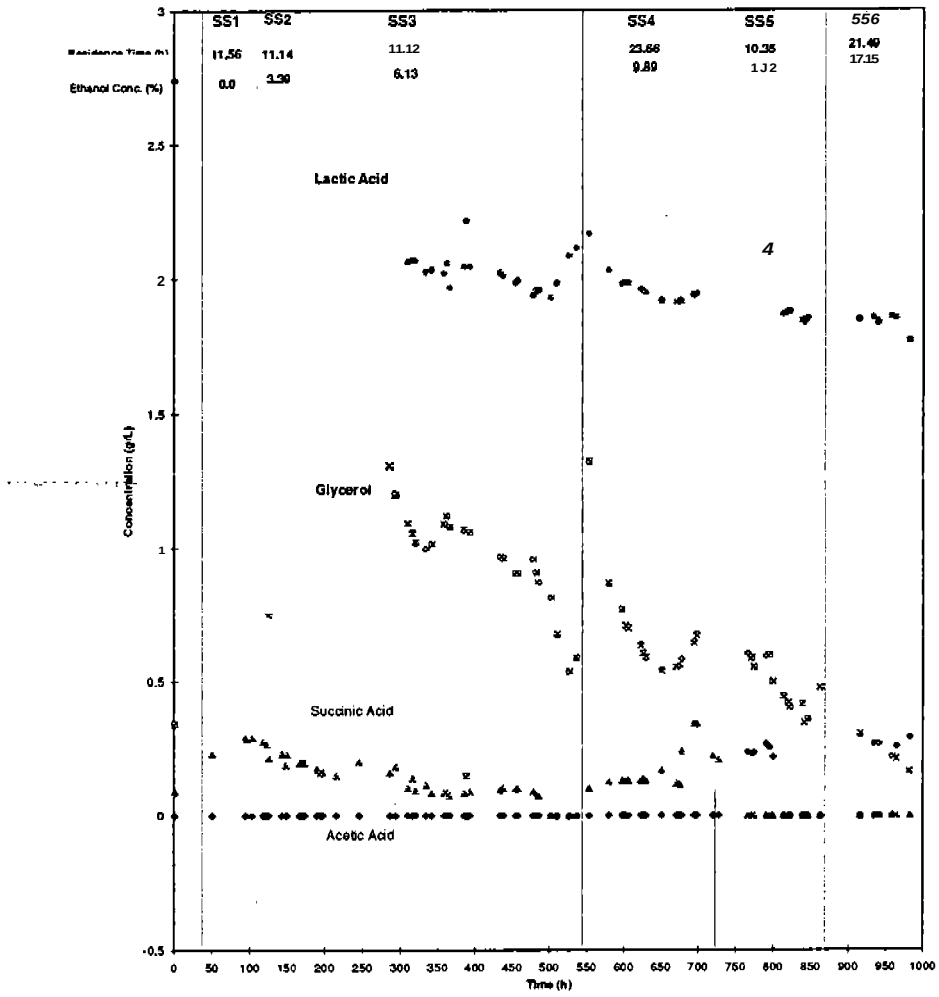
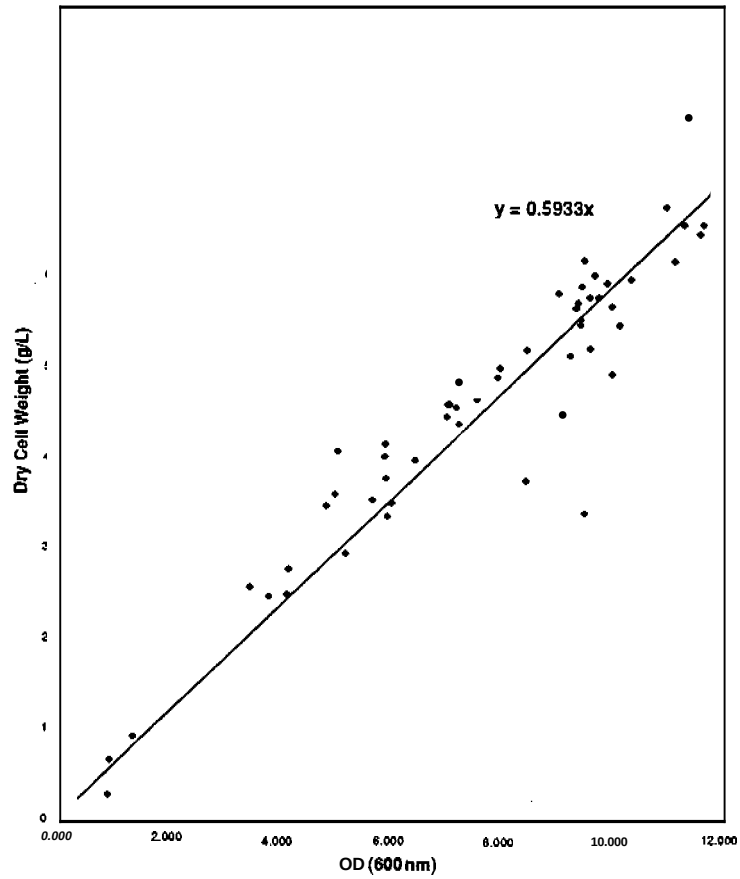


Figure 4
Dry Cell Weight and Optical Density Correlation



Observations on **Ethanol in** the **Fermentor Off-gas**
Phase II AMOCO **CRADA** Report
Nancy Combs 6/12/95

Introduction

This is an informal discussion on the presence of ethanol in the fermentor off-gas. These observations were made when a 1L batch fermentation was *run* with a flask containing YPD medium attached to the fermentor exhaust line. It **was** noted that ethanol was accumulating in the off-gas flask and is important to note for scale-up purposes. **This** write-up discusses the fermentation run conditions **and** the data generated from the **run.**

Discussion

A 2L Bioflo III fermentor was set up as a batch fermentation with *Saccharomyces* ***cerevisiae*** D_5A on 1% w/v yeast extract, **2%** w/v peptone and 2% w/v glucose (YPD). **A** 1L working volume was used. the temperature **was** set at **37** °C, pH **was** controlled at 5.0, **and** the broth was agitated at **150** rpm and aerated at 1.0 vvm. **A** 2L flask **with** 1L YPD was aeseptically attached **to** the off-gas line so that the fermentor exhaust would sparge into the medium. **Part** of the data generated from this fermentation included ethanol concentrations in the off-gas **flask.** It **was** noted that the ethanol was increasing in ***the*** **flask** over the 24 hour period to over 2 g/L. The fermentor concentration only reached **9** g/L (see Table 1). Some of the increase in ethanol concentration in the off-gas flask was due to evaporation, but even after 8 hours. 1 g/L had accumulated in the flask. The Bioflo III condenser was cooled with cold tap water, not chilled water and the condenser was not packed with materials that increase surface area. The loss of ethanol seen at the 1L scale could be significant at larger scale if the condenser system is not very efficient. **Mass** spectrophotometer data **from** the PDU 9000L fermentors is important. especially considering there ***are*** no condensers on the fermentors at this time. in determining ethanol loss from the fermentor exhaust.

Table **1. Raw data from a** *Saccharomyces cerevisiae* D_5A batch fermentation **on** YPD medium. **The** fermentation was **run** at **37** °C, pH 5.0, **1** vvm aeration, and **150** rpm agitation.

Fermentation Time **(hours)**	Sample	**Glucose (g/L)**	**Ethanol (g/L)**
T=O	Fermentor	18.5	0.985
	Off-gas flask	21.1	0.002
T = 8.5	Fermentor	0.0	9.06
	Off-gas **flask**	22.9	1.18
T = 24	Fermentor	0.0	**5.62**
	Off-gas flask	**29.1**	***2.67***